Nomogram for Prediction of Pathologic Complete Remission Using Morphometric Change and Biomarker Expression in Rectal Cancer After Preoperative Chemoradiotherapy

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Directed by Professor Nam Kyu Kim

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ABSTRACT

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Numerous molecular markers and imaging tools have been studied to predict pathologic complete remission (pCR) after preoperative chemoradiotherapy (CRT) for rectal cancer. However, none of these has not shown definite outcomes. The aim of this study is to develop a nomogram to predict pCR by analyzing relevant biomarkers and endoscopic findings. Tumor specimens have been collected prospectively from 120 patients before preoperative CRT in patients with rectal cancer between November 2011 and April 2014. All

patients underwent curative resection with total mesorectal excision at 8 weeks after completeness of preoperative CRT. Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis, mRNA expression levels of seven candidate biomarkers (p53, p21, Ki-67, VEGF, CD133, CD24, CD44) were evaluated from fresh tumor samples before CRT. The expression of mRNA was indicated with ΔCt by correction according to the expression of GAPDH (target Ct – GAPDH Ct). The relative quantity of mRNA in pathologic complete remission (pCR) tissue to that in non-pCR tissue was calculated from the relative ratios of $2^{-\Delta Ct}$ between two conditions. Lower ΔCt and Higher $2^{-\Delta Ct}$ mean higher expression of mRNA. Endoscopic evaluation has been done pre- and post-preoperative CRT. Clinical complete remission by endoscopic finding was no visualization of tumor, white scar, and red scar. Clinical variables were also evaluated. Univariate and multivariate logistic regression analysis with clinical and biologic variables were used to make a predictive model for pCR. Nomogram was developed in a training set (n=80) and validated in external validation set (n=40). Both discrimination and calibration were measured by the area under a receiver operating characteristic (ROC) curve (AUC) and calibration plot, respectively. The pCR was shown in 24 patients (30%). Among seven biomarkers, the mRNA expression levels of

four biomarkers (p53, p21, Ki67, CD133) significantly correlated with pCR in training set. Patients showing low expression of p53 and/or high expression of p21, Ki67, CD133 exhibited a significantly greater pCR rate. Among 27 patients showing endoscopic clinical complete response (cCR) after preoperative CRT, 17 patients (63.0%) demonstrated pCR. Lower tumor location showed a higher pCR rate than middle tumor [19 (38.8%) vs. 5(16.1%), p = 0.031]. By logistic regression analysis, tumor location, endoscopic finding after preCRT and four biomarkers (p53, p21, Ki67, CD133) were significantly correlated with pCR. Based on the multivariate prediction model with these variables, a nomogram were drawn for prediction for pCR, and which showed good discrimination ability in training set (AUC=0.945) and validation set (AUC=0.922). The calibration plot demonstrated good agreement between actual and predicted pCR in both patient set. The nomogram for prediction of pCR may be useful in treatment decisions after preoperative CRT to select complete responders for a wait-and-see policy or sphincter preserving surgery.

Key words : rectal cancer, chemoradiotherapy, pathologic complete remission, biomarkers, endoscopy, prediction nomogram.

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I. INTRODUCTION

Most of expert groups as well as treatment guideline have adopted a preoperative chemoradiotherapy (CRT) as preferred method to improve oncologic outcomes in patients with advanced middle and low rectal cancer. In these patients, CRT is expected to cause tumor downstaging and to allow performing a sphincter-saving procedure with negative circumferential resection margin. The ideal effect of CRT is complete response when tumor is

totally replaced by scar. The rate of pathologic complete remission (pCR) after preoperative CRT varies between 12-16% in standard CRT[1-3] and can increase up to 24-30% with advanced regimens and target chemotherapy agents.[4] Patients with significant response to CRT are expected to have better oncologic outcomes, and those with pCR potentially might even avoid major surgery.[5]

Several biomarker models are being tested for prediction of CRT response with conflicting results. To date a number of tumor proteins are being studied as candidate predictive markers that can be used to predict response to CRT in rectal cancer. These proteins are involved in carcinogenesis in different ways – apoptosis, tumor proliferation, angiogenesis, mismatch repair and their expression can easily be assessed by immunohystochemical (IHC) analysis or reverse transcriptase polymerase chain reaction (RT-PCR). Previously we described a scoring system based on IHC analysis of four tumor proteins (p53, VEGF, p21 and Ki67) that accurately predicted pCR in middle and low rectal cancer.[6] However IHC lacks of reproducibility and quantitative assessment of gene expression.

Gross tumor characteristics detected by endoscopy have been suggested for assessment of tumor response after preoperative CRT. It was suggested that

regularly scheduled reassessments might provide a safe alternative to patients with endoscopic findings of clinical complete response.

Each predictive modality has its own strength and weakness; therefore, there have been several attempts to develop models or nomograms to predict the treatment response of preoperative CRT. A combination of two or more modalities provided complementary information about treatment response and yielded higher accuracy and specificity than the individual investigations. The combination of morphological and functional imaging with the numerous potential molecular markers and identified genes will provide comprehensive information on each individual patient and make possible individualized treatment therapy.

The aim of this study was to develop a prediction nomogram of pCR to preoperative CRT in rectal cancer based on mRNA expression of tumor proteins measured by RT-PCR and endoscopic findings.

II. MATERIALS AND METHODS

1. Patients

Total 120 consecutive patients with rectal cancer were enrolled prospectively

between November 2011 and April 2014. Following inclusion criteria were used: histologically proven primary rectal adenocarcinoma, clinically staged as $T_2N_{(+)}M_0$ or $T_{3\text{-}4}N_{any}M_0$ planned for preoperative CRT and curative resection.

Eighty patients were assigned to the training set for development of the nomogram for prediction of pCR. For external validation of the nomogram, 40 patients assigned to the validation set in sequence of treatment time.

All the patients underwent preoperative staging (clinical T- and N-staging) with the use of magnetic resonance imaging (MRI) reviewed by single radiologist and endorectal ultrasound (EUS) performed by a single surgeon. MRI can evaluate circumferential margin and pelvic lymph node status more accurately. So, in case of discrepancies in T- or N-staging between MRI and EUS, we used MRI for clinical staging. Clinical N(+) stage was stated if regional lymph nodes were found to be larger than 10 mm or had spiculated shape.

After inclusion and before starting preoperative CRT all patients underwent rigid sigmoidoscopy and two-punch biopsy from visibly the deepest area of tumor, which was further used for reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Low rectal cancer was stated if lower tumor edge was located < 5 cm from the anal verge by rigid sigmoidoscopy, and middle

rectal cancer if it was at 5-10 cm from the anal verge.

Endoscopy was performed before preoperative CRT and 4 weeks after completion of preoperative CRT. We defined the endoscopic findings of tumor after preoperative CRT and evaluated treatment response.

The study was approved by the Institutional Review Board (IRB) for the protection of human subjects at the Severance Hospital, Yonsei University College of Medicine.

2. Preoperative chemoradiotherapy and Surgery

All the patients received preoperative CRT as indicated by the multidisciplinary team decision. Radiation therapy was delivered using dual-photon linear accelerators at an energy level of 6-MV/10-MV. Long-course radiation therapy included 25 fractions 1.8Gy each delivered to the pelvis over a period of 5 weeks (5 days per week), resulting in 45Gy total radiation dose that was followed by the 5.4 Gy boost targeting the primary tumor.

Chemotherapy was administered to all patients with two types of regimen options, 5-fluorouracil with leucovorin or xeloda only. Two cycles of intravenous bolus injection of 5-fluorouracil (425 mg/m^2/day) and leucovorin (20 mg/m^2/day) were administered for 5 days during the first and fifth weeks

of radiation therapy. Xeloda was continuously administered orally at a dose of 1450 mg/m2/d twice daily during the radiation therapy period.

All the patients completed the preoperative CRT in full and underwent curative surgery at 8 weeks after completion of CRT.

3. Quantitative real-time PCR

Total RNA was isolated from fresh samples and used for RT-PCR. We extracted total RNA with an RNeasy plus mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. We synthesized cDNA using a Transcriptor first strand cDNA synthesis kit (TaKaRa Bio Inc., Otsu, Japan) and 1 μg of total RNA. We conducted real-time polymerase chain reaction (PCR) in duplicate using TaqMan master mix (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems viia7 Real-Time PCR System. Seven biomarkers (P53, P21, Ki67, VEGF, CD133, CD24, CD44) were chosen as candidates from our previous research findings[6] and published data, and mRNA expression levels were investigated. We normalized the mRNA expression levels of TP53 (assay ID Hs01034249_m1), CDKN1A (assay ID Hs00355782_m1), MKI67 (assay ID Hs01032443_m1), VEGFA (assay ID Hs00900055_m1), PROM1 (assay ID Hs01009250_m1), CD44

(assay ID Hs01075861_m1) and CD24 (assay ID Hs03044178_g1 to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Hs02758991_g1) housekeeping gene. We performed relative quantification using the QuantStudio software v1.2 (Applied Biosystems). Expression level of each protein was measured as ΔCt and $2^{-\Delta Ct}$ compared to expression of GAPDH (ΔCt = target Ct – GAPDH Ct). Lower ΔCt and higher $2^{-\Delta Ct}$ means the higher expression of each protein.

4. Endoscopic findings

We ventured a hypothesis that no visualization of tumor, white scar, or red scar would be associated with “cCR” and ulcerations and remaining masses of any size would be associated with "non-cCR” (Figure 1). The endoscopic findings were interpreted by two independent endoscopists blinded to the patients’ clinical information. The consistency of the endoscopic findings between the two independent observers was greater than 90%. In cases of disagreement, the endoscopic finding was determined by consensus.

5. Assessment of tumor response to CRT and histopathology

All of the operation specimens were reviewed by a single pathologist that

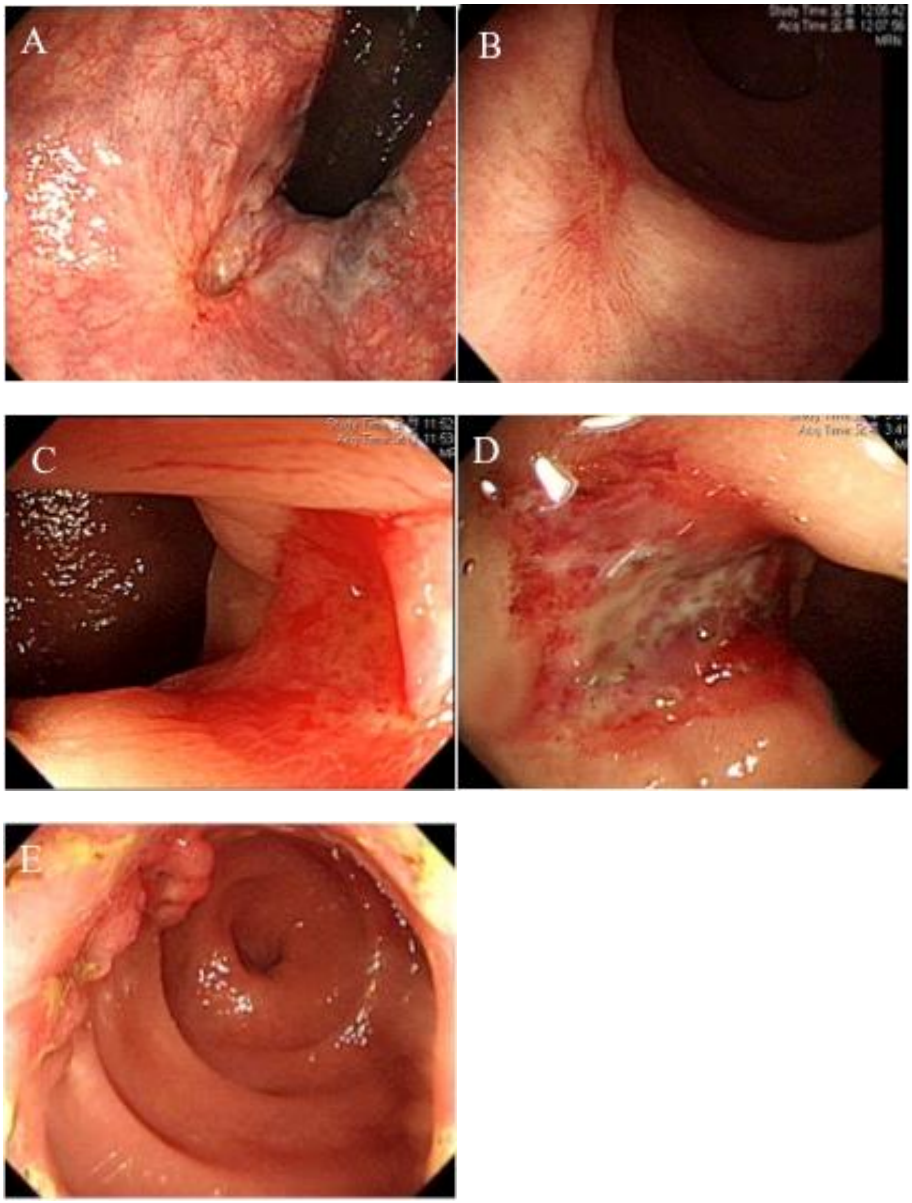

Figure 1. Various endoscopic findings of primary tumors after preoperative chemoradiotherapy.

(A. no visualization; B. white scar; C. red scar; D. ulceration; E. remaining mass).

was blinded to patient clinical information. Staging was performed according to the 7th American Joint Committee on Cancer (AJCC) TNM staging manual. T-downstaging was observed if pathologic T-stage (pT) was smaller compared to clinical T-stage (cT) in the same patient. In patients who initially presented with clinically positive lymph nodes [cN(+)], N-downstaging was defined as conversion to pathological lymph node negative status [pN(-)].

The tumor response to preoperative CRT was evaluated using the Mandard's tumor regression grade (TRG). Tumors classified as TRG1 or TRG2 were considered to be responders, whereas tumors staged as TRG3-5 were defined as nonresponders. Pathologic complete remission (pCR) was stated if no viable tumor was found (TRG1) with no lymph node involvement (pN0).

6. Statistical analysis

We built the nomogram using logistic regression model to discriminate between pCR and non-pCR in training set (n = 80). After development of the nomogram, validation set (n = 40) was used for validity verification in external patients cohort.

Categorical data were analyzed using Fisher's exact or χ^2 tests. Continous variables were analyzed using two-sided *t*-test or Mann-Whitney test.

Univariate logistic regression analysis were used first to assess the association between each variable and pCR and to select variables, which entered following multivariate logistic regression analysis. The final multivariate model was chosen on the basis for the pCR prediction model and tested by Hosmer-Lemeshow goodness of fit test for evaluation of prediction ability. Based on the prediction model with identified predictive factors, a nomogram were drawn for prediction of pCR. The model performance was quantified in terms of the discrimination and calibration performance. Discrimination is the predictor's ability to separate patients between pCR and non-pCR. Discrimination ability was meseared by the area under a receiver operating characteristic (ROC) curve. A higher ROC area under the curve (AUC) indicates a better discriminatory power. An AUC of 0.50 indicates that the test is good as random chance for discriminating an outcome, whereas an AUC of 1.0 indicates perfect discrimination of the rest (sensitivity and specificity of 100%). In general, the model is considered relatively good for values above 0.75. Calibration is the agreement between actual probability and predicted probability of pCR produced by the model. This was evaluated with a calibration curve, where patients were grouped by predicted pCR and then plotted as actual versus predicted pCR. We used the bootstrapping resampling

method (300 repetitions) to obtain relatively unbiased estimates and to check internal validation. Both discrimination and calibration were evaluated in the training and the validation set, respectively. All statistical analyses were performed using SPSS software version 20 (SPSS, Chicago, IL, USA) and R software version 3.0.1 (the R foundation for statistical computing, Vienna, Austria). Values of $p<0.05$ were considered statistically significant.

III. RESULTS

1. Patients characteristics and clinical variables

A total of 120 consecutive patients were included in this trial. Detailed patient characteristics in training and validation set are listed in Table 1.

Before starting treatment, the majority of patients in training set presented with advanced T-stage and N-stage: cT3-T4 stage was diagnosed in 97.5% and cN(+) – in 81.2% of patients (Table 1).

In training set, pathologically confirmed tumor downstaging after preoperative CRT was found in 60.0% (48/80) of patients for T-stage and in 57.5% (46/68) of patients for N-stage. Good response to preoperative CRT (TRG1 and TRG2) was observed in 40.0% of patients, and pCR was revealed

Table 1. Patient characteristics

Characteristics	Training (n=80)	Validation (n=40)	*p*
Median age, years (range)	60 (36-86)	64 (37-86)	0.497
Sex			0.791
Male	50 (62.5)	24 (60.0)	
Female	30 (37.5)	16 (40.0)	
Median distance from AV, cm (range)	5.3 (1-11)	5.3 (1-10)	0.923
Tumor location			0.895
Middle	31 (38.8)	16 (40.0)	
Low	49 (61.3)	24 (60.0)	
Median Pre CRT CEA, ng/mL (range)	8.28 (0.5-51.8)	8.13 (0.9-46.2)	0.944
Initial tumor differentiation			0.576
G1/G2	13 (16.3)/60 (75.0)	4 (10.0)/31 (77.5)	
G3/G4	6 (7.5)/1 (1.3)	5 (12.5)/0 (0.0)	
Clinical T and N stage			0.846
cT2N+	2 (2.5)	1 (2.5)	
cT3N-/N+	14 (17.5)/54 (67.5)	10 (25.0)/25 (62.5)	
cT4N-/N+	1 (1.2)/9 (11.2)	0 (0.0)/4 (10.0)	
Endoscopy			0.781
cCR	26 (32.5)	12 (30.0)	
non-cCR	54 (67.5)	28 (70.0)	
Pathological T and N stage			0.960
ypT0N-	24 (30.0)	11 (27.5)	
ypT1N-/N+	6 (7.5)/1 (1.2)	2 (5.0)/0 (0.0)	
ypT2N-/N+	12 (15.0)/3 (3.8)	8 (20.0)/1 (2.5)	
ypT3N-/N+	16 (20.0)/16 (20.0)	7 (17.5)/9 (22.5)	
ypT4N+	2 (2.5)	2 (5.0)	
TRG			0.987
TRG1/TRG2	24 (30.0)/16 (20.0)	11 (27.5)/9 (22.5)	
TRG3/TRG4	28 (35.0)/12 (15.0)	14 (35.0)/6 (15.0)	
Operation			0.955
Low anterior resection	56 (70.0)	29 (72.5)	
uLAR with CAA	20 (25.0)	9 (22.5)	
APR	4 (5.0)	2 (5.0)	

AV, anal verge; CEA, carcinoembryonic antigen; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; G4, undifferentiated; cCR, clinical complete response; TRG, tumor regression grade; uLAR, ultra low anterior resection; CAA, coloanal anastomosis. Unless indicated otherwise, numbers in parenthesis are percentages.

in 30.0% of patients. The clinical and pathologic characteristics of patients in validation set are similar with training set (Table 1).

Table 2. Univariate analysis of clinical variables associated with pCR and TRG in training set (n = 80).

Characteristics		pCR		*p*	TRG		*p*
		Yes (n=24)	No (n=56)		TRG1-2 (n=40)	TRG3-4 (n=40)	
Age	≤60	14 (33.3)	28 (66.7)	0.494	18 (42.9)	(57.1)	0.179
	>60	10 (26.3)	28 (73.7)		22 (57.9)	16 (42.1)	
Sex	Male	13 (26.0)	37 (74.0)	0.313	25 (50.0)	20 (50.0)	1.000
	Female	11 (36.7)	19 (63.3)		15 (50.0)	15 (50.0)	
Tumor location (cm)	Low (1-5)	19 (38.8)	30 (61.2)	0.031	29 (59.2)	20 (40.8)	0.039
	Middle (>5)	5 (16.1)	26 (83.9)		11 (35.5)	20 (64.5)	
Pre CRT CEA (ng/mL)	≤5	17 (34.7)	32 (65.3)	0.249	27 (55.1)	22 (44.9)	0.251
	>5	7 (22.6)	24 (77.4)		13 (41.9)	18 (58.1)	
Clinical T stage	cT2/cT3	20 (28.6)	50 (71.4)	0.477	33 (47.1)	37 (52.9)	0.176
	cT4	4 (40.0)	6 (60.0)		7 (70.0)	3 (30.0)	
Clinical N stage	cN(-)	6 (40.0)	9 (60.0)	0.363	8 (53.3)	7 (46.7)	0.775
	cN(+)	18 (27.7)	47 (72.3)		32 (49.2)	33 (50.8)	
Histology	G1/G2	22 (30.1)	51 (69.9)	1.000	37 (50.7)	36 (49.3)	1.000
	G3/G4	2 (28.6)	5 (71.4)		3 (42.9)	4 (57.1)	
Endoscopy	cCR	18 (69.2)	8 (30.8)	<0.001	23 (88.5)	3 (11.5)	<0.001
	non-cCR	6 (11.1)	48 (88.9)		17 (31.5)	37 (68.5)	

pCR, pathologic complete response; TRG, tumor regression grade; CEA, carcinoembryonic antigen; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; G4, undifferentiated; cCR, clinical complete response. Unless indicated otherwise, numbers in parenthesis are percentages.

Univariate analysis of different clinical variables in training set revealed that tumor location in lower rectum was significantly associated with pCR and TRG response. Tumors located in lower rectum demonstrated significantly higher rate of pCR (38.8%, 19/49) and TRG1-2 (59.2%, 29/49) than middle rectal tumors (16.1%, 5/31 and 35.5%, 11/31, p=0.03 and 0.039, respectively). Endoscopic findings demonstrated a significant correlation between clinical

CR and pCR or TRG1-2 (Table 2).

2. mRNA expression and responses to preoperative CRT

In training set, the level of expression value of the studied biomarkers mRNA was not significantly different between patients who did and did not develop T- or N-downstaging. But mRNAs of four biomarkers (p53, p21, Ki-67 and CD133) were expressed differently between patients who had a pCR and those who had not develop a pCR, as well as between responders to CRT (TRG 1-2) and non-responders (TRG 3-4). Responders to CRT and patients with a pCR demonstrated significantly higher ΔCt and lower $2^{-\Delta Ct}$ of p53 mRNA, but lower ΔCt and higher $2^{-\Delta Ct}$ of mRNA of p21, Ki-67 and CD133 (Table 3).

3. Nomogram for prediction of pCR

In univariate and multivariate logistic regression analysis in training set, tumor location, endoscopic finding, low expression of p53 mRNA and high expression of p21, Ki67, CD133 mRNA was significantly associated with pCR (Table 4). These variables were selected for further analysis as possible predictors of pCR to CRT.

Prediction models for pCR using logistic regression analysis were suggested

Table 3. The relative quantity of biomarkers mRNA in tumor tissue according to ypTNM, T- and N-downstaging, TRG and pCR in trainage set (n = 80).

Biomarker	Expression value	ypTNM			T-downstaging			N-downstaging			TRG			pCR		
		Stage 0, I (n=42)	Stage II,III (n=38)	*p*	Yes (n=48)	No (n=32)	*p*	Yes (n=46)	No (n=22)	*p*	TRG1-2 (n=40)	TRG3-4 (n=40)	*p*	Yes (n=24)	No (n=56)	*p*
p53	ΔCt (mean±SD)	6.35±1.54	6.00±1.15	0.266	6.39±1.83	6.07±2.42	0.511	6.45±1.91	5.60±2.47	0.122	6.65±1.81	5.87±2.28	0.042	6.97±1.95	5.96±2.08	0.047
	$2^{-\Delta Ct}$ (mean±SD)	1.74±1.51	1.84±1.24	0.720	2.78±2.68	7.27±4.76	0.213	2.91±2.86	10.04±6.2	0.159	1.52±1.16	7.64±0.97	0.038	1.31±1.03	5.98±1.20	0.036
p21	ΔCt (mean±SD)	6.09±1.71	6.31±1.06	0.493	6.04±1.63	6.31±1.19	0.413	6.16±1.36	6.28±1.36	0.732	5.98±1.64	6.31±1.27	0.048	5.58±1.71	6.39±1.30	0.045
	$2^{-\Delta Ct}$ (mean±SD)	1.48±1.99	0.82±0.52	0.053	1.49±1.04	0.89±0.72	0.069	1.25±0.88	0.97±0.80	0.506	1.57±0.19	0.93±0.08	0.039	2.13±2.67	0.87±0.72	0.033
Ki67	ΔCt (mean±SD)	8.91±3.19	10.01±3.98	0.175	9.48±3.73	9.63±3.98	0.856	9.55±4.25	9.48±3.70	0.950	8.62±3.38	10.46±4.0	0.043	8.16±2.77	10.13±4.05	0.033
	$2^{-\Delta Ct}$ (mean±SD)	1.35±1.37	0.86±0.90	0.056	3.41±2.12	1.41±0.55	0.489	3.74±2.49	1.38±0.38	0.509	4.54±0.67	0.69±0.05	0.006	6.05±2.71	1.14±2.05	0.002
VEGF	ΔCt (mean±SD)	5.91±1.86	6.19±2.01	0.521	6.00±1.83	6.09±2.10	0.840	6.22±1.89	5.65±2.01	0.259	5.71±1.87	6.37±1.95	0.130	5.68±1.82	6.19±1.97	0.272
	$2^{-\Delta Ct}$ (mean±SD)	2.78±4.93	2.36±3.91	0.680	2.55±1.64	2.63±1.20	0.932	2.22±1.18	3.17±1.73	0.406	3.03±0.91	2.14±0.94	0.374	3.22±0.72	2.31±0.80	0.403
CD133	ΔCt (mean±SD)	8.52±2.15	9.03±1.68	0.246	8.73±2.35	9.13±2.07	0.440	8.98±2.33	8.92±2.27	0.919	8.36±2.05	9.42±2.32	0.034	8.13±2.26	9.22±2.17	0.046
	$2^{-\Delta Ct}$ (mean±SD)	1.71±1.66	1.09±1.30	0.066	2.22±1.98	1.50±1.40	0.358	1.85±0.51	1.89±0.81	0.958	2.50±0.27	1.37±0.26	0.045	3.28±0.30	1.35±0.03	0.036
CD24	ΔCt (mean±SD)	3.97±1.26	3.87±1.30	0.740	4.01±1.21	3.79±1.37	0.452	3.89±1.18	3.81±1.54	0.829	3.90±1.18	3.94±1.37	0.899	3.91±1.21	3.92±1.31	0.973
	$2^{-\Delta Ct}$ (mean±SD)	1.65±1.67	1.77±1.72	0.748	1.57±0.59	1.92±0.83	0.368	1.67±0.61	2.06±0.07	0.401	1.68±0.67	1.73±0.71	0.890	1.72±0.90	1.70±0.59	0.959
CD44	ΔCt (mean±SD)	4.89±1.74	4.90±1.01	0.986	4.93±1.63	4.84±1.08	0.795	4.82±1.16	4.71±1.07	0.726	4.81±1.73	4.98±1.06	0.613	5.00±2.09	4.85±1.05	0.659
	$2^{-\Delta Ct}$ (mean±SD)	1.13±0.72	1.00±0.72	0.425	1.08±0.69	1.06±0.76	0.934	1.09±0.71	1.16±0.85	0.699	1.18±0.72	0.96±0.70	0.181	1.14±0.71	1.04±0.72	0.605

TRG, tumor regression grade; pCR, pathologic complete remission.

Table 4. Logistic regression analysis of clinical variables and biomarker expression in tumor tissue by RT-PCR for assessment of pCR in training set (n = 80).

variable	univariable			multivariable		
	OR	95% CI	*p*	OR	95% CI	*p*
Age	0.993	(0.953, 1.034)	0.722			
Gender	1.648	(0.621, 4.369)	0.315			
BMI (kg/m^2)	1.003	(0.862, 1.167)	0.967			
AV_distance	0.807	(0.641, 1.017)	0.070			
Tumor_location (cm)	0.304	(0.099, 0.927)	0.036	0.086	(0.005, 1.491)	0.042
Pre_CRT_CEA (ng/mL)	0.995	(0.950, 1.041)	0.816			
Clinical_Tstage	0.388	(0.023, 6.518)	0.511			
Clinical_Nstage	0.574	(0.179, 1.846)	0.352			
Histology_grade	1.545	(0.381, 6.265)	0.543			
Endoscopic_finding	18.00	(5.481,59.112)	0.000	134.676	(7.616,2381.642)	0.001
p53 ΔCt	1.713	(1.148, 2.556)	0.008	2.19	(0.775, 6.189)	0.039
p53 $2^{-\Delta Ct}$	0.55	(0.331, 0.914)	0.021	0.972	(0.207, 4.556)	0.071
p21 ΔCt	0.598	(0.404, 0.885)	0.010	0.769	(0.207, 2.855)	0.095
p21 $2^{-\Delta Ct}$	2.281	(1.281, 4.062)	0.005	3.75	(0.497, 28.277)	0.020
Ki67 ΔCt	0.655	(0.480, 0.894)	0.008	0.384	(0.126, 1.169)	0.042
Ki67 $2^{-\Delta Ct}$	1.922	(1.247, 2.961)	0.003	0.395	(0.086, 1.805)	0.023
VEGF ΔCt	0.868	(0.675, 1.116)	0.270			
VEGF $2^{-\Delta Ct}$	1.044	(0.943, 1.155)	0.406			
CD133 ΔCt	0.627	(0.447, 0.880)	0.007	1.289	(0.401, 4.147)	0.040
CD133 $2^{-\Delta Ct}$	1.637	(1.177, 2.278)	0.003	3.283	(0.998, 10.796)	0.035
CD24 ΔCt	0.993	(0.680, 1.451)	0.972			
CD24 $2^{-\Delta Ct}$	1.008	(0.758, 1.339)	0.959			
CD44 ΔCt	1.076	(0.779, 1.488)	0.656			
CD44 $2^{-\Delta Ct}$	1.194	(0.616, 2.312)	0.600			

BMI, body mass index; AV, anal verge.

with these six clinical and biological variables (Table 5). Of three prediction

Table 5. Logistic regression model using biomarker expression in tumor tissue by RT-PCR and endoscopic finding for assessment of pCR in training set (n = 80).

Model 1

Varaible	OR	lower	upper	*p*
p53deltaCT	1.948	1.212	3.547	0.013
p21deltaCT	0.537	0.312	0.848	0.014
Ki67deltaCT	0.671	0.434	0.905	0.034
CD133deltaCT	0.799	0.51	1.197	0.296
AUC	0.859375			

Model 2

Variable	OR	lower	upper	*p*
p53deltaCT	2.101	1.186	3.722	0.011
p21deltaCT	0.518	0.307	0.874	0.014
Ki67deltaCT	0.726	0.512	1.03	0.073
CD133deltaCT	0.787	0.515	1.202	0.268
Tumor location	0.314	0.074	1.337	0.117
AUC	0.875744			

Model 3

Variable	OR	lower	upper	*p*
p53deltaCT	1.717	1.039	3.263	0.053
p21deltaCT	0.439	0.193	0.799	0.019
Ki67deltaCT	0.642	0.384	0.9	0.027
CD133deltaCT	0.594	0.318	1.002	0.069
Endoscopic	39.228	6.989	399.305	<.0001
Tumor location	0.356	0.047	2.063	0.271
AUC	0.9449405			

models according to the including variables, model 3 with all six variables demonstrated the highest AUC and was chosen. The Hosmer-Lemeshow goodness of fit test was not significant (p = 0.984), indicating good fit of this model. This prediction model was visually represented by a nomogram to

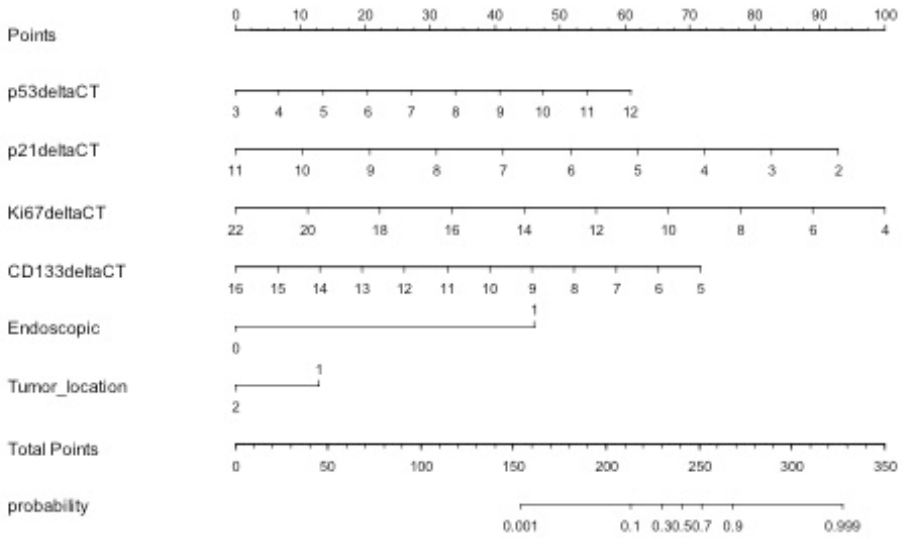

Figure 2. Nomogram predicting the probability of pathologic complete remission (pCR) after preoperative chemoradiotherapy in rectal cancer. The nomogram is used by totaling the points identified on the top scale for each independent six variables. The total points projected to the bottom scale indicate the % probability of pCR.

predict the probability of pCR (Figure 2). To use the nomogram, a vertical line is drawn up to the top point row to assign points for each variable. Then the total number of points is calculated, and a vertical line is drawn downwardsfrom the total point row to obtain the probability of pCR. According to nomogram, patients with 210, 240, 270 total points had estimated pCR probability of 10, 50, 90 %, respectively. Figure 3 demonstrates ROC curve and calibration plot of training set. Area under the ROC curve of

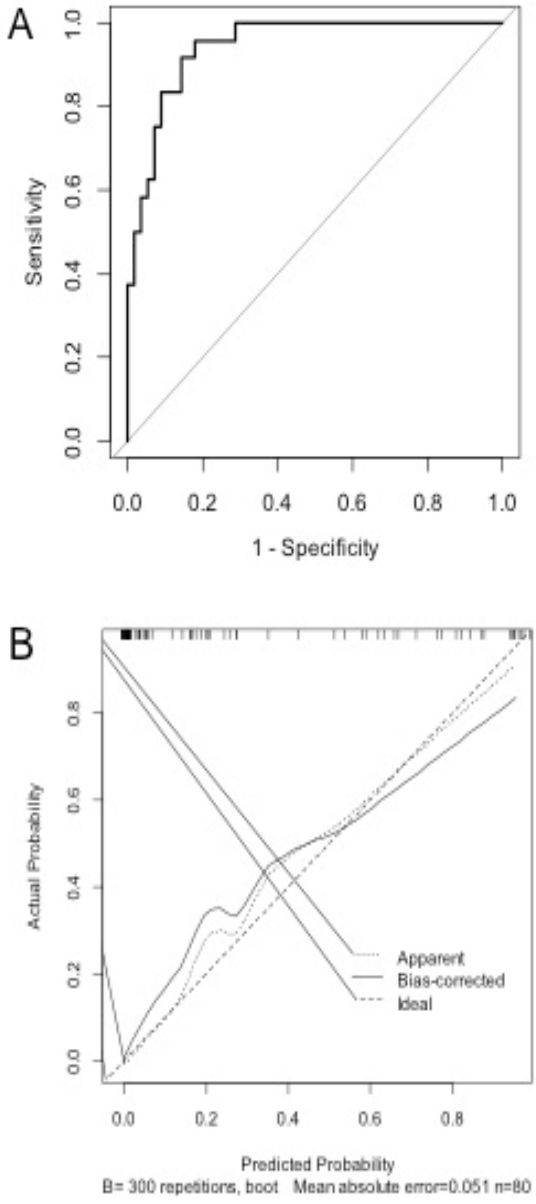

Figure 3. Discrimination and calibration of nomogram in training set (n = 80). A. Receiver operating characteristic (ROC) curve by the multiple logistic model. Area under the ROC curve is 0.945 [95% confidence interval (CI): 0.900-0.989]. B. Calibration plot for probability of pCR. Predicted and actual pCR probabilities are plotted as logistic calibration (bootstrap 300 repetitions).

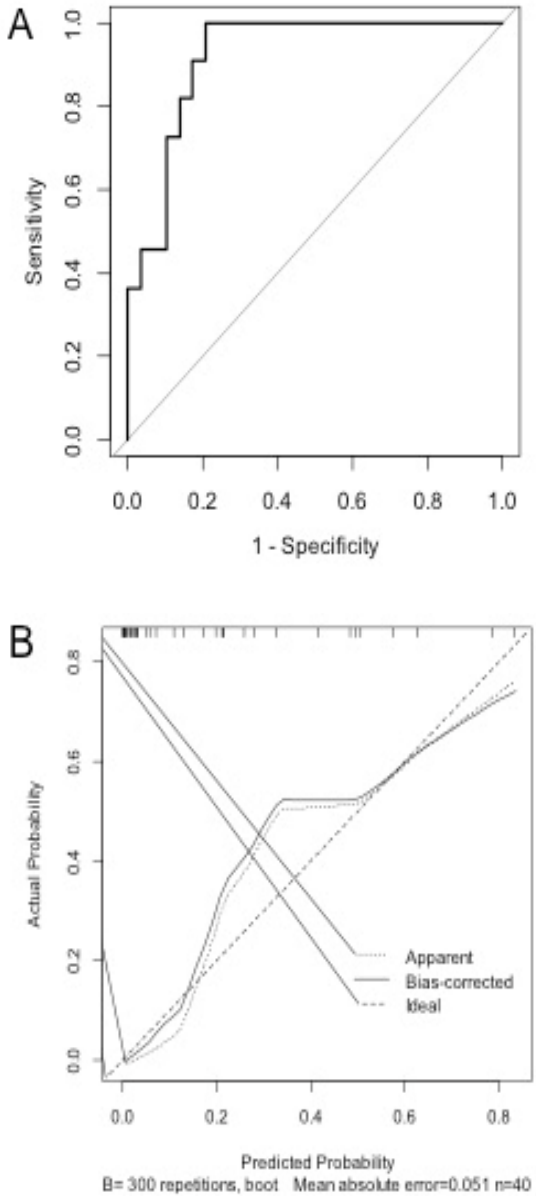

Figure 4. Discrimination and calibration of nomogram in external validation set (n = 40). A. Receiver operating characteristic (ROC) curve by the multiple logistic model. Area under the ROC curve is 0.922 [95% confidence interval (CI): 0.841-0.999]. B. Calibration plot for probability of pCR. Predicted and actual pCR probabilities are plotted as logistic calibration (bootstrap 300 repetitions).

the multivariate model was 0.945 [95% confidence interval (CI): 0.900–0.989]. The calibration plot showed good agreement between predicted and actual probability. In the external validation set, the AUC was 0.922 [95% confidence interval (CI): 0.841–0.999]. The calibration plot of predicted and actual probability showed good correlation in low and high probability area. However, in middle probability area, it seems not to show a good correlation, because the intermediate probability could be ambiguous for binary variable (Figure 4).

IV. DISCUSSION

Various modalities have been studied and proposed to assess and predict responses to CRT. For morphologic assessment of tumor response after preoperative CRT, endoscopic findings and imaging studies, including magnetic resonance imaging (MRI) and positron emission tomography (PET), have been used and demonstrate good results. Clinical factors and serum carcinoembryonic antigen (CEA) have also been investigated and shown to hold some predictive value. Notwithstanding, due to the limitations of these modalities, many molecular markers have been assessed for evaluation and

prediction of tumor response to preoperative CRT in patients with rectal cancer. More than 40 different biomarkers have been explored in the literature, with conflicting results in predicting the outcomes of CRT. Molecular biomarkers analyzed using immunohistochemistry and gene expression profiling have been investigated and may play a possible role as predictive models for tailored treatment of patients undergoing preoperative CRT.

In the present study, we developed the nomogram combining endoscopic findings of morphometric tumor change and mRNA expression levels of four biomarkers (p53, p21, Ki67 and CD133) by quantitative RT-PCR to predict pCR in rectal cancer patients receiving preoperative CRT. These predictive model were externally validated and showed a good model performance in terms of calibration and discrimination. We believe that this comprehensive nomogram is useful for pCR prediction and would be the guidance for individualized treatment.

Unlike other works in this field that evaluated prognostic value of biomarkers in rectal cancer, in this study we developed a prediction system that is based on evaluation of mRNA expression of 4 biomarkers and endoscopic finding in a complex. This prediction model can be utilized after completion of CRT in rectal cancer patients to predict a pCR. Previously we reported on a prognostic

scoring system that utilized the level of expression of p53, VEGF, p21 and Ki67 measured by IHC analysis.[6] These proteins were chosen from a panel of 12 markers as having a significant correlation between their expression level and pCR in 81 patients. One of the major limits of that study was implementing IHC analysis to evaluate protein expression in tumor samples. Although this technique is wide-spread and easily available, still its major disadvantage is low reproducibility. Hence for present study we employed real-time RT-PCR as a more reliable, sensible and reproducible method that allows quantification of gene expression.[7] Although the number of studied patients is rather small, one of the strengths of this study is its prospective nature. In contrast to our previous study all consecutive patients in the study period were included thus overcoming the influence of selection bias inherent in retrospective studies. In addition, we added the endoscopic finding as clinical tool for evaluation of morphometric changes of tumor after preoperative CRT. Gross tumor response findings would be the valuable assessment modality for prediction of pathologic tumor response. In our study, we could demonstrate the importance of endoscopic evaluation of primary tumor after preoperative CRT for rectal cancer.

Still, the observed pCR rate (30%) is higher than reported by most groups

worldwide. This partly can be related to a higher proportion of patients with tumors below 5 cm from anal verge (61.3%). Among studied clinical factors tumor location in low rectum was found to be the only significant predictor of good response to CRT and pCR in the present research. It is difficult to rule out any evident reasons for this phenomenon. One of the possible theoretical explanations to higher sensitivity to CRT of low rectal tumors compared to middle rectal tumors is that they might be better vascularized and faster growing. The quicker cell cycle may influence the sensitivity and response rate to CRT. The second possible explanation is that low rectal tumors can be biologically different from higher ones, thus it can be reflected in differences in biomarkers profile between them. We are not aware of any evidence-based data that can support or refute this idea. But if this result is not accidental this may open a way to further research in the field of finding biologic differences between low and high rectal tumors.

Another possible limitation of this study is that the samples were collected and analyzed only before starting CRT. We didn't study tumor samples after completion of CRT or after surgery, so we didn't evaluate possible changes in biomarkers mRNA expression that could be induced by CRT. However, the major aim of this study is to predict pCR after CRT and provide the useful

information in making treatment strategies after CRT. The developed predictive model is based on RT-PCR analysis of a group of biomarkers analyzed together but not independently. This may help to get a more realistic profile of the tumor and better predict its response to CRT.

The selection of p53, VEGF, p21 and Ki67 as candidate biomarkers for the present study was based on our previous research findings[6] and published data that supports the correlation between expression of these proteins and rectal cancer sensitivity to CRT.

Among abovementioned tumor proteins p53 is probably the most extensively studied biomarker in this field. Protein p53 plays an important role in genetic stability, cell proliferation, apoptosis and inhibition of angiogenesis, and in a number of studies the presence of p53 mutations was found to correlate inversely with pCR, although not in all of them this association was significant.[6,8-22] Twelve of these studies[9-18] used IHC staining to identify mutant p53 and only in two of them, including our previous research[6,16] a significant correlation was demonstrated between strong mutant p53 staining in pretreatment tumor biopsies and resistance to CRT. The group of Spitz et al.[16] was able to show that lack of p53 staining is strongly correlated with response to CRT ($p=0.02$) and strong p53 staining is related to residual cancer

in regional lymph nodes (p=0.02). Other 4 studies utilized RT-PCR to measure the expression of this biomarker,[19-22] and three of them,[8,20,21] like our present study, demonstrated lower prevalence of p53 mutation in patients with pCR. Among them only the group of Rebischung et al.[21] revealed a significant correlation between low expression of p53 and pCR. The results of our present investigation confirm the findings from our previous research[6] and other research groups[16,21] that low expression of mutant p53 is predictive of tumor response to CRT and a pCR.

Another biomarker for apoptosis is p21 that can inhibit cell cycle through p53-dependent and p53-independent pathways, so presumably tumor cells expressing p21 would be more prone to chemotherapy agents and radiation. Its clinical significance in rectal cancer is still not well described as controversial data is reported to date. Earlier reports[9,10] failed to confirm association between expression of p21 and prognosis whereas later reasearch[17,23] demonstrated this link. Our present work is consistent with our previous IHC study[6] and showed that high expression of p21 is strongly associated with pCR. Conversely in the paper of Sim et al. high IHC staining of p21 in tumors before starting and after 7 days of CRT was significantly correlated with non-pCR and decreased disease free survival.

One of the biomarkers of high proliferating activity of tumor cells is Ki67. Earlier its higher expression on IHC analysis was found to be correlated with response to radiation only,[24] but not to CRT[25] in rectal cancer. The findings of our present work and previous study[6] demonstrate significant correlation between high expression of Ki67 and pCR. This suggests that proliferating tumors may be more sensible to a long course CRT.

Besides abovementioned four biomarkers we added to the initial panel three cancer stem cell proteins (CD133, CD24, CD44) that are most often described as having prognostic value in colorectal cancer. Cancer stem cell has the characteristics of resistance to chemotherapy and radiotherapy. Therefore, there have been some efforts to investigate the correlation of cancer stem cell markers with the treatment response to CRT. CD133 is probably the most studied marker of colorectal cancer stem cells. A number of research groups[26-31] reported correlation between CD133 expression and clinicopathologic features and oncologic outcomes in rectal cancer patients, while others couldn't demonstrate this association.[32] CD133 expression means the existence of cancer stem cell and high level is correlated with resistance to CRT. In contrast, high expression of CD133 mRNA demonstrated better response to CRT and higher pCR rate in our study. The level of CD24 was also

found to be significantly associated with CRT response in some reports. Huh et al. revealed that, among 13 molecular markers, only elevated CD44 mRNA level in pretreatment biopsies was predictive of poor tumor regression, and CD133 level had no significant correlation with CRT response. Until now, there is no confirmative result that cancer stem cell marker is predictive of CRT response and useful in clinical field. More investigation is needed to develop predictive model using cancer stem cell markers.

Habr-Gama et al. attempted to provide a clear definition of complete clinical response (cCR) after preoperative CRT using endoscopic features[9]. They defined the positive and negative signs for cCR. Positive signs for cCR frequently included whitening of the mucosa, presence of any telangiectasia, subtle loss of pliability of the rectal wall harboring the scar, and no gross evidence of residual tumor. In contrast, positive signs of residual disease included residual deep ulceration, superficial ulcer irregularity, palpable nodule, and significant stenosis.

We ventured a hypothesis that no visualization of tumor, white scar, or red scar would be associated with “cCR” and ulcerations and remaining masses of any size would be associated with "non-cCR.” Of the 26 patients that demonstrated endoscopic cCR, 18 (69.2%) showed pCR. Of the 54 patients

that demonstrated endoscopic non-cCR, 48 (88.9%) showed non-pCR. For assessment of pathologic complete responses, endoscopic findings exhibited 75.0% sensitivity and 85.7% specificity. Endoscopic findings were significantly correlated with tumor response after preoperative CRT for rectal cancer.

To date, there have been several attempts to develop models or nomograms to predict the outcomes of CRT. Van stiphout et al. suggested a nomogram predicting pCR for locally advanced rectal cancer based on clinical features and early sequential ^{18}F-FDG PETCT imaging. The pCR rates were 21.4% in training set (n = 112) and 23.1% in validation set (n = 78). The selected predictive features for pCR were cT-stage, cN-stage, response index of SUVmean and maximal tumor diameter during treatment. The model performances (AUC) were 0.78 (training) and 0.70 (validation). The high probability group for pCR resulted in 100% correct predictions for training and 67% for validation.

Jwa et al. assessed a nomogram to predict ypN status after preoperative CRT in rectal cancer. The nomogram was developed in a training cohort (n = 891) using logistic regression analyses and was validated in a separate cohort (n = 258). Patient age, preoperative CRT tumor differentiation, cN stage, ypT stage,

lymphovascular invasion, and perineural invasion were reliable predictors of LN metastasis after preoperative CRT and were used for the construction of the nomogram. The nomogram showed good discrimination ability in training and validation cohort. The calibration plot suggested good agreement between actual and predicted LN status after preoperative CRT.

In present study, prediction model consisting of tumor location, endoscopic finding, and four biomarkers expression demonstrated a highly value to discriminate between pCR and non pCR. We produced a nomogram for prediction of pCR using six variables and could be valuable system in clinical field.

Comparing to IHC the RT-PCR approach is less time consuming and more reliable and endoscopic morphometric change can be easily accessible. The prediction model demonstrated high discrimination and calibration abilities in identifying patients who are prone to develop a pCR and thus to have a better outcome and possibly to avoid major surgery. But before implementing this prediction model to practice it should be tested and validated on other large independent patient cohort. This process is planned as the next step in our work.

Our prediction nomogram demonstrated good performance of discrimination

and calibration abilities in training set. However, despite good discrimination ability in validation set, calibration plot did not perfectly show good agreement. The calibration plot showed good correlation in low and high probability of pCR but not in middle probability area. This prediction nomogram may be useful in patients with low or high probability, but for the patients with intermediate scores, we need more reliable and substantial prediction model.

As shown in our study, a combination of clinical and biologic variables provided complementary information about treatment response and yielded higher accuracy and specificity than the individual investigations. The combination of morphological imaging and the numerous potential molecular markers will provide comprehensive information on each individual patient and make possible individualized treatment therapy. The prediction of complete tumor remission has been regarded as important, because it impacts clinical decisions for treatment strategy. If complete remission of rectal cancer after preoperative CRT can be predicted, radical surgery which results in increasing postoperative morbidity and poor quality of life with stoma can be avoided. In addition, local excision or wait-and-see treatment strategies can be recommended if the tumor shows an excellent tumor response to preoperative CRT for rectal cancer.

There are still controversies regarding outcomes of predictive modalities of treatment response after preoperative CRT in rectal cancer. Combined models may be the future trend for predicting treatment responses. The ability to predict pathological tumor response before treatment will significantly impact patient selection for preoperative CRT and can potentially modify treatment strategies.

V. CONCLUSION

The present research demonstrated the benefit of developed prediction model in prediction of pCR in patients with rectal cancer. Our prediction nomogram encompasses clinical and biological factors including tumor location, endoscopic finding, and 4 biomarkers, studied in a combination that helped to achieve high sensitivity and sufficient accuracy in prediction of a pCR. This finding stresses the importance of evaluation the mechanisms of cancer sensitivity to CRT as a complex, but not as separate processes of tumor cells proliferation, apoptosis, angiogenesis and others. We used the combination of biomarkers and morphometric findings that showed distinct patterns of expression and features between pCR and non-pCR to include in the prediction nomogram. Despite certain limitations of this study its major

advantage is demonstrating the utility of prediction nomogram in identifying patients more prone to be pCR and making treatment strategies.

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ABSTRACT (IN KOREAN)

직장암의 수술 전 화학방사선요법 후 형태계측 변화와 바이오마커 발현을 이용한 병리학적 완전관해 예측 노모그램

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허혁

직장암의 수술 전 화학방사선요법 치료 후 병리학적 완전관해를 예측하기 위해 수 많은 분자 수준의 표지자와 영상학적 도구들이 사용되어 왔다. 하지만 종양의 치료 반응 평가에 대한 명확한 결과를 보여주는 방법은 없었다. 본 연구의 목적은 관련 바이오마커 및 내시경 소견을 분석함으로써 병리학적 완전관해를 예측하는 노모그램을 제시하는 것이다. 종양 검체는 2011년 11월에서 2014년 4월 사이 수술 전 화학방사선요법을 시행 받기 전의 직장암 환자 120명으로부터 전향적으로 채취되었다. 모든 환자는 수술 전 화학방사선요법 종료 후 8주 뒤에 전직장간막 절제술로 근치적 수술을 받았다. 역전사 중합효소 연쇄반응 (RT-PCR) 분석을 통하여 신선 종양 검체로부터 7개의 바이오마커 (p53, p21, Ki-67, VEGF, CD133, CD24, CD44)의

mRNA 발현 수준을 평가하였다. mRNA의 발현 정도는 GAPDH의 발현 정도에 따라 교정 (목표 Ct - GAPDH Ct)하여 ΔCt로 나타내었다. 병리학적으로 완전관해를 보이지 않은 검체의 mRNA에 대한 완전관해를 보인 검체의 mRNA의 상대적인 양은 두 검체의 $2^{-\Delta Ct}$ 값의 상대비로 계산하였다. 낮은 ΔCt와 높은 $2^{-\Delta Ct}$ 값은 mRNA의 발현 수준이 높다는 것을 의미한다. 내시경 검사는 선행 항암방사선 치료 전과 후(선행 항암방사선 치료 종료 후 4주 뒤)에 실시하였다. 내시경 검사를 통해 임상적으로 완전 관해를 판단한 기준은 육안적으로 종양이 보이지 않고, 백색 반흔 혹은 적색 반흔이 남아있는 경우로 하였다. 임상적 변수 또한 평가하였다. 병리학적 완전관해 예측모델을 구축하기 위해 임상변수 및 생물학적 변수를 로지스틱 회기 모형을 이용하여 단변량 및 다변량 분석을 시행하였다. 80명의 훈련 집합 (training set)에 대하여 노모그램 (Nomogram)을 개발하고 40명의 외부 검증 집합 (validation set)에서 검증을 시행하였다, 식별력과 검정력은 곡선하면적 (area under the curve, AUC)과 눈금측정평면도 (calibration plot)을 이용해 측정하였다. 병리학적 완전관해는 24명 (30%)의 환자에서 관찰되었다. 7개의 바이오마커 중, 4개의 바

이오마커 (p53, p21, Ki67, CD133)의 mRNA 발현 수준은 병리학적 완전관해와 유의한 상관관계가 있었다. P53의 낮은 발현 및 또는 p21, Ki67, CD133의 높은 발현을 보이는 환자들에서 병리학적 완전관해율이 유의하게 높았다. 수술 전 화학방사선요법 후 시행한 내시경 검사 상 임상적인 완전관해를 보인 27명의 환자 가운데 병리적 완전관해를 보인 환자는 17명 (63.0%)이었다. 종양의 위치가 낮은 환자들이 종양이 직장의 중간부에 위치한 환자에 비하여 더 높은 병리적 완전관해율을 보였다 [19 (38.8%) vs. 5(16.1%), p = 0.031]. 로지스틱 회기모형을 통해 종양의 위치, 수술 전 화학방사선요법 후의 내시경 소견, 4 개의 바이오마커 (p53, p21, Ki67, CD133)가 병리적 완전관해와 유의하게 상관관계가 있음을 확인하였다. 4 개의 바이오마커, 수술 전 화학방사선요법 후의 내시경 소견 및 종양의 위치를 이용한 다변량 예측모델을 기반으로 구축한 노모그램 (nomogram)은 훈련 집합 (AUC=0.945) 및 검증 집합 (AUC=0.922) 내에서 양호한 식별력을 보였다. 눈금측정평면도 (calibration plot)는 두 집합 모두에서 실제 병리학적 완전관해와 예측된 병리학적 완전관해의 확률이 유의하게 일치하는 것을 보여주었다. 병리학적 완전관해를 예측하기

위한 노모그램은 수술 전 화학방사선요법 후 병리학적 완전관해를 보여 수술 없이 경과 관찰을 하거나 괄약근 보존수술을 시행할 수 있는 대상환자를 선별하는데 도움을 줌으로써 치료 방침 결정에 매우 유용하게 사용 될 수 있을 것이다.

핵심되는 말 : 직장암, 화학방사선요법, 병리학적 완전관해, 바이오마커, 내시경, 예측 노모그램.